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ORIGINAL PAPER

In situ expression of trehalose synthesizing genes, *TPS1* and *TPPB*, in *Arabidopsis thaliana* using the GUS reporter gene

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Abstract Two transgenic *Arabidopsis* lines were derived that expressed promoter regions for either *AtTPS1* (At1g78580) or *AtTPPB* (At1g78090) using constructs containing the β-glucuronidase (GUS) reporter gene. These two genes function in tandem to produce the disaccharide, trehalose, and they likely have important regulatory and signaling functions in higher plants. Both genes were expressed nearly constitutively in *Arabidopsis* and expression was high in younger tissue and typically diminished with age. Similar expression patterns for both promoters were observed in etiolated and in light grown seedlings. Dense expression of both genes was observed during germination on day 1 but expression was absent from hypocotyls 3 days later. In contrast to *AtTPS1*, the expression of *AtTPPB* was concentrated in the root meristem of 7-day old light grown seedlings. The expression of both *AtTPS1* and *AtTPPB* was mainly observed in young, actively dividing tissues, such as the shoot apex, and in flower parts including anthers, pistils, siliques and developing seeds. Expression of both genes also was clearly associated with vascular bundles, the root-hypocotyl junction, the pedicel-silique junction and related structures involved in bulk solute transport. Transcript levels of *AtTPS1* and *AtTPPB* were either repressed or were little affected by exogenous sucrose, glucose, fructose or trehalose when measured by quantitative real-time PCR. However, both trehalose biosynthesis genes were induced two to tenfold by sorbitol, mannitol and NaCl. Responses of *AtTPS1* and *AtTPPB* to the same chemical and stress treatments were not detected by changes in GUS activity. This may be due to the stability of the GUS protein relative to transcript levels. Because *AtTPS1* and *AtTPPB* function in tandem to produce trehalose it was not surprising that the expression of both genes was distributed similarly in *Arabidopsis* tissues.

Keywords Trehalose synthesis · Gene expression · Promoter region · Plant development · Molecular signaling

Abbreviations

GUS	β-glucuronidase
QPCR	Quantitative real-time polymerase chain reaction
TPPB	Trehalose 6-phosphate phosphatase B
TPS1	Trehalose-6-phosphate synthase 1

Introduction

Trehalose (1,1 α-D glucopyranosyl α-D-glucopyranoside) is a nonreducing disaccharide that is widespread in microbes, plants and insects (Eastmond and Graham 2003). Trehalose functions both as a storage carbohydrate and as a stress protectant in many organisms (Wingler 2002) but its

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function in higher plants is less clear. Desiccation tolerant plants including the club moss, *Selaginella lepidophylla*, the leafy liverwort, *Plagiochila asplenoides*, and the flowering plant, *Myrothamnus flabellifolia*, contain trehalose (Suleiman et al. 1979; Drennan et al. 1993). Consequently, the function of trehalose in these species was associated with stress mitigation. Trehalose is normally only present in trace amounts in higher plants, except in the presence of validamycin A, a fungicide that inhibits cleavage of the disaccharide (Wingler 2002; Goddijn and van Dun 1999). Genetic disruption of the trehalose biosynthetic pathway prevented embryo maturation in *Arabidopsis* (Eastmond et al. 2002) and there is evidence that trehalose or related compounds in the trehalose pathway function in molecular signaling (Vogel et al. 1998; Avonce et al. 2004; Paul et al. 2008).

Three enzymes are responsible for trehalose metabolism in higher plants (Goddijn and van Dun 1999). Trehalose-6-P is formed from Glc-6-P and uridine-5′-diphosphoglucose by the action of trehalose 6-P synthase (TPS). Subsequently, trehalose-6-P is dephosphorylated by trehalose-6-P phosphatase (TPP) yielding the free sugar. The hydrolysis of trehalose occurs by α-trehalase, a substrate specific α-glucosidase (Müller et al. 2001; Goddijn et al. 1997). An analysis of the *Arabidopsis* genome revealed that 11 putative *TPS* genes were present (Leyman et al. 2001) and based on results of yeast complementation studies only one of these, *AtTPS1* (At1g78580), was shown to be functional in trehalose synthesis (Blázquez et al. 1998; Vogel et al. 2001). According to Leyman et al. (2001) and Lunn (2007) class I *TPS* genes (*TPS1-4*) are capable of synthesizing trehalose-6-P, whereas class II genes (*TPS5-11*) do not. Note that *TPS* genes (*TPS2-4*) are present in very low concentrations in *Arabidopsis* (Paul et al. 2008). In addition, class II *TPS* genes contain a phosphatase domain and are responsive to plant hormones and environmental stress (Ramon et al. 2009). Among 10 putative *TPP* genes in *Arabidopsis* only two genes, *AtTPPA* and *AtTPPB*, were functional in complementation studies (Vogel et al. 1998). Therefore, the expression of *AtTPS1* and *AtTPPB* was examined in the present study because it has clearly been established that both genes are translated and synthesize active proteins in *Arabidopsis*.

Several prior studies analyzed the developmental expression of genes involved in trehalose metabolism in *Arabidopsis* by detecting transcripts with PCR or hybridization based techniques. Blázquez et al. (1998) originally reported that the expression of *AtTPS1* was low and not organ specific. Conversely, van Dijken et al. (2004) showed via QPCR that *AtTPS1* was expressed similarly in rosette leaves and stems, was induced by illumination and was expressed at low levels in roots. This same laboratory further demonstrated that *AtTPS1* expression was up-regulated during seed development. Almeida et al. (2007) used immunogold to identify the intracellular location of *AtTPS1* in transgenic tobacco. These authors observed gold labeling in vacuoles, chloroplasts, cell walls and sieve elements and suggested that TPS metabolism was important in sugar signaling and transport.

Similar to At*TPS1,* transcripts of *AtTPPA* and *AtTPPB* were strongly expressed in developing flowers and in young seedlings of *Arabidopsis* but there was little or no expression of either gene in leaves or stems of mature plants (Vogel et al. 1998). Results of the *Arabidopsis* microarray database (http://www.arabidopsis. org/links/microarrays.jsp), further indicated that transcripts involved in trehalose biosynthesis, including those for *AtTPS1* and *AtTPPB*, were expressed at low concentrations in this species (see van Dijken et al. 2004). Paul et al. (2008) examined the tissue specific expression of 22 *Arabidopsis* genes involved in trehalose metabolism and found that the expression of *AtTPS1* and *AtTPPB* was largely constitutive.

The objective of the current study was to monitor the expression of *AtTPS1* and *AtTPPB* throughout plant development using *Arabidopsis* transformed with the Promoter::GUS reporter gene constructs. In contrast to various QPCR results cited above, van Dijken et al. (2004) examined the expression of *AtTPS1* using a Promoter::GUS reporter gene and based solely on GUS activity only observed this gene in developing embryos. Watkinson (2002) transformed *Arabidopsis* with a *TPS* promoter::GUS fusion construct using an *Arabidopsis* gene (*AtTPS8*, At1g70290). Expression was not observed in leaves or stems but weak expression was observed in roots, root hairs, flowers, anthers and stamens. Interestingly, GUS expression was strongly induced by wounding, which is consistent with the suggestion that class II *TPS* genes are highly regulated and often respond to environmental stimuli (Paul et al. 2008; Ramon et al. 2009).

The above studies clearly demonstrated that transcripts for specific genes involved in trehalose metabolism were expressed in *Arabidopsis* but often at very low levels. Also, these experiments broadly identified organs involved in the expression of genes regulating trehalose biosynthesis. However, the specific location of *AtTPS1* and *AtTPPB* within individual organs has not been studied in detail except in developing embryos. This is an important deficiency because these two genes are directly involved in trehalose and trehalose 6-P synthesis and trehalose 6-P, in particular, has been linked to molecular signaling by Glc, ABA and cold stress (Avonce et al. 2004). Our goal was to gain insights into the function of trehalose in higher plants by determining the location of gene expression within various tissues by using Promoter::GUS fusions. We show that *AtTPS1* and *AtTPPB* were expressed in nearly all plant

organs throughout development but expression was most evident in young, rapidly dividing tissues, in the shoot apex, in floral organs and was strongly associated with vascular structures and related tissues involved in solute transport.

Materials and methods

Plant materials

Seeds of *Arabidopsis* [*Arabidopsis thaliana* (L.) Henyh Columbia ecotype (Col 0)] were normally sown on a solid culture medium. Transformed seeds and appropriate controls initially were sterilized with one-third strength commercial bleach for 10 min and were then washed five times with sterile H_2O. The sterilized seeds were sown on 0.8% agar containing full strength Murashige-Skoog (MS) basal salts plus vitamin B5 and 0.1 mM MES (pH 5.7). Seeds were incubated at 4°C for 2 days and were then grown with continuous fluorescent light at 22°C and 150 μmol m^{-2} s^{-1} photosynthetically active radiation in a growth cabinet. Etiolated seedlings were propagated similarly but were maintained in darkness for 7 days at 22°C. Alternatively, unsterilized seeds were sown directly on soil and intact plants were grown in controlled environment chambers with continuous illumination (150 μmol m^{-2} s^{-1}), a temperature of 22°C and with watering, fertilization and other cultivation details as reported previously (Bae and Sicher 2004).

To obtain liquid cultures seedlings approximately 50 sterilized *Arabidopsis* seeds were grown in 50-mL sterile plastic Falcon tubes filled with 5-mL of liquid media containing MS basal salts and vitamin B5. Seedlings were grown with continuous shaking at 200 rpm in a growth cabinet with temperature and light as described above. Chemical and stress treatments were imposed after 7 days growth. Sufficient amounts of sucrose (Suc), glucose (Glc), fructose (Fru), trehalose or NaCl were added to the liquid cultures to yield 0.1 M final concentrations. Mannitol and sorbitol were added similarly but final concentrations for the polyols were 0.25 M. An equal volume of sterile water was used as a control. Treated seedlings and controls were incubated for an additional 24-h with continuous shaking prior to harvesting. Cold-treatments were at 4°C for 24-h with light and continuous shaking as described above. At the end of the treatment period seedlings were filtered through steel sieves, washed briefly with distilled water and rapidly transferred to liquid N_2 to stop metabolism. Samples were extracted immediately or were frozen for up to 3 weeks at −80°C. The liquid cultured samples were used for RNA extraction and QPCR analysis as described below.

Promoter::GUS fusion constructs, transformation and GUS analysis

The *AtTPS1* (At1g78580) and *AtTPPB* (At1g78090) promoter regions were amplified from *Arabidopsis* genomic DNA using the following primer sets: ATpTPSf/ATpTPSr, and ATpTPPf/ATpTPPr (Table 1). The amplified *AtTPS1* promoter contained a 3,147-bp fragment located upstream from the start codon, which contained *Eco*RI/*Nco*I restriction sites in the 5′ and 3′ regions, respectively. The amplified *AtTPPB* promoter contained *Eco*RI/*Bgl*II restriction sites and was a 3,027 bp fragment also located upstream from the start codon. Using the restriction enzyme sites cited above the amplified promoter regions were inserted into the binary vector, pCAMBIA 1305. The *AtTPS1* promoter or *AtTPPB* promoter::*GUS* fusion products were created by replacing the 35S promoter region in the original vector. After assemblage the vectors were transformed into *Agrobacterium tumefaciens* strain C58CI by freeze-thaw transformation (Hofgen and Willmitzer 1988). Control transformations also were performed with the unmodified binary vector containing the 35 s promoter. Subsequently, the floral dip method was used to transform *Arabidopsis* after the developing shoots were removed to induce a second phase of bolting (Clough and Bent 1998). The T1 seeds were germinated on selective MS media containing 50 μg mL^{-1} hygromycin and the resultant lines with active GUS expression were advanced to the T3 or T4 generation and were shown to be homozygous. Successful insertion of the promoter GUS::fusion constructs was confirmed by Southern blot analysis. Overall transformation efficiencies were 1–3% and we obtained at least three lines for each promoter capable of GUS expression. One line of each transformant was chosen at random for subsequent experiments.

In situ GUS assays were performed as described elsewhere (Rodrigues-Pousada et al. 1993). Plants were grown either in liquid culture or on soil as described above. Whole *Arabidopsis* seedlings and specific organs were fixed in 90% ice cold acetone for 15 min and then washed with a rinse solution containing 50 mM phosphate buffer (pH 7.2), 0.5 mM $K_3Fe(CN)_6$ and 0.5 mM $K_4Fe(CN)_6$. Tissues were then stained in the rinse solution with 2 mM of the cyclohexylammonium salt of 5-bromo-4-chloro-3-indoyl α-D-glucopyranoside, (X-Glc, Gold Biotechnology, St. Louis, MO). The samples were vacuum infiltrated for 5 min to remove air bubbles and were then incubated for 24 h in the staining solution at 37°C. After staining the samples were washed with an aqueous ethanol dehydration series to remove chlorophyll (15, 30, 50, 70, 85, 95, 100 and 100%). Samples with GUS expression were observed and photographed with a Nikon model SMZ1500 dissecting microscope equipped with digital imaging capability.

Table 1 *Arabidopsis* primer sequences used for promoter amplification and for performing quantitive real time-PCR analysis (QPCR)

Name (AGI #)	Sequence (5′–3′)	Expected size (bp)
ATpTPS	F: CGGAATTCATCGCGATGATCGTCTATTG	3147
(At1g78580)	R: CATGCCATGGACGCTCACACCAAAACAGAAC	
ATpTPP	F: CGGAATTCGATCTCTCCCAAGTCTAG	3027
(At1g78090)	R: GAAGATCTATTCTCGTTGAGACAGAGAG	
AtTPS1	F: CTCTAATGCATCAGTTGATGTTGTC	206
(At1g78580)	R: GAAGTTCTGGTTCGAAGAAAGTGTA	
AtTPPB	F: CTTTCATAACCCATGAGATG	199
(At1g78090)	R: AGCACTCTTTCATTACTCTG	
AtTRE	F: GGGACTATACATGAGAAGCTCAAAG	237
(At4g24040)	R: CCACTACGATCAAATCTACCAAAGT	
AtTEF1	F: CGTTGCTGTTGGTGTTATTAAGAG	204
(At5g60390)	R: GAGGGAGAGAGAAAGTCACAGAAAT	

F, forward primer; *R*, reverse primer

Quantitative real-time PCR

A quantitative real-time RT-PCR (QPCR) technique was used to measure *AtTPS1*, and *AtTPPB* transcript levels in response to various chemical and stress treatments as described above. Samples were ground to a fine powder under liquid nitrogen in a mortar and pestle and total RNA was isolated using an RNeasy mini kit (Qiagen, Valencia, CA) with an optional RNase-free DNase treatment. First strand cDNA was synthesized with 1 μg of total RNA and oligo $(dT)_{20}$ primers using SuperScript III RNase H Reverse Transcriptase (Invitrogen, Carlsbad, CA). The resultant cDNA was diluted fivefold and was used as a template for real-time QPCR. Amplifications were performed with an Mx3005P® QPCR System using Brilliant® SYBR® Green QPCR Master Mix (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Primers were designed using the Primer 3 program of Biology WorkBench (http://workbench.sdsc.edu/). Real-time QPCR reactions contained 12.5 μL of 2X Brilliant SYBR® Green QPCR Master Mix, 5 μL of fivefold diluted cDNA, 2,500 nM gene-specific primer and 300 nM reference dye in a final volume of 25 μL. The default thermal profile for the QPCR reactions was 95°C for 10 min, 40 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 30 s. In addition, a dissociation (melting) curve was generated for each primer set at the end of the amplification reaction. The minimum threshold was manually set to 0.1. This value was used to determine the minimum number of cycles (C_T) required for the SYBR® Green fluorescent signal (ΔRn) to cross the threshold value. Three replications were performed with three different biological samples to obtain means and standard errors for the C_T values. A house keeping gene, translation elongation factor 1-α (*EF-1-α*, At5g60390), was used as an expression control. To calculate PCR efficiencies (E) for each primer, dilution curves were generated with five points over a threefold dilution. E values were calculated as $E = [10^{(-1/\text{slope})}] - 1$ (Pfaffl 2001). To compare data from different reactions the C_T values for all genes of interest (C_{TGOI}) were normalized to the C_T values of *EF-1-α* (C_{TTEF}). Expression ratios (treatment/control) were calculated using the equation $\left[(E_{GOI})^{\Delta C}_{TGOI}/(E_{TEF})^{\Delta C}_{TTEF}\right]$.

Results

Developmental expression of *AtTPS1* and *AtTPPB*

The expression of *AtTPS1* and *AtTPPB* was examined at various developmental stages and in different tissues using transformed plants that possessed *AtTPS1* or *AtTPPB* promoter::*GUS* reporter gene fusion constructs (Figs. 1, 2, 3, 4). Although there were exceptions, similar patterns of expression were detected for both *AtTPS1* and *AtTPPB* throughout *Arabidopsis* development. Weak expression of both *AtTPS1* and *AtTPPB* was observed in imbibed seeds on day 0 (Fig. 1a, b), whereas strong expression was obtained with the 35S promoter on this date (Fig. 1c). The expression of GUS was absent from the seed coat of the *AtTPS1* transformant immediately after radicle emergence on day 1 (Fig. 1d). However, weak GUS activity was observed on the seed coat of the AtTPPB transformant on this date (Fig. 1e). High levels of expression for both *AtTPS1* and *AtTPPB* also were found throughout 1-day old light-grown seedlings (Fig. 1f, g). Discrete expression patterns for both genes in specific tissues became visible in 2-days and 3-days old light-grown seedlings (Fig. 2a–d). Unlike at the earlier growth stage, the expression of both *AtTPS1* and *AtTPPB* was not detected in hypocotyl tissues

of 3-days old light-grown seedlings. In addition, expression of both genes was concentrated around the vascular tissue in cotyledons, in the shoot apex and in the root immediately adjacent to the hypocotyl. This expression pattern for both genes was continued for 5, 7 and 18-days old seedlings (Fig. 2e–j). However, by 7-days after germination the expression of both *AtTPS1* and *AtTPPB* was detected in stem tissue and, this was primarily observed in association with the vascular tissue. In 21-days old seedlings GUS expression for both genes was detected diffusely in leaves and in the stem but expression was clearly associated with vascular tissues (Fig. 2k, l). It was also obvious by this stage that GUS expression for both *AtTPS1* and *AtTPPB* was more concentrated in younger than in older leaves. Both genes were highly expressed in the shoot apex of 21-day-old seedlings. Control experiments performed with 21 s-old seedlings transformed with the 35S promoter::GUS construct, showed that all plant parts were uniformly expressed GUS activity at this developmental stage (Fig. 2m). Additionally, untransformed, *Arabidopsis* tissues were color free in control experiments (data not shown).

The expression of *AtTPS1* and *AtTPPB* was closely associated with root vascular tissues of 7-days old light grown seedlings (Fig. 3a, b). However, unlike *AtTPS1*, the expression of *AtTPPB* was clearly visible in the root apex at this developmental stage. GUS expression for both promoters was observed in cotyledons and in the shoot apex of 7-days-old dark-grown seedlings (Fig. 3c, d). Again the expression of both promoters was associated with vascular tissue in etiolated seedlings. GUS expression in etiolated tissue was greater for *AtTPPB* than *AtTPS1*. The in situ expression of *AtTPS1* and *AtTPPB* also was investigated using mature plants that were grown in soil for 5–6 weeks. The expression of *AtTPS1* and *AtTPPB* was observed throughout young leaves (Fig. 3e, f) and was observed in trichomes (Fig. 3g, h).

Both *AtTPS1* and *AtTPPB* were strongly expressed in floral organs (Fig. 4a, b). Both genes were expressed in sepals, particularly in and around veins, but GUS expression for both promoters was absent from petals. Both *AtTPS1* and *AtTPPB* were strongly expressed in the floral receptacle. The expression of *AtTPS1* and *AtTPPB* also was observed in the stamen (Fig. 4c, d), at the distal and proximal ends of the pistil (Fig. 4e, f), in cauline leaves (not shown) and in roots of mature plants (not shown). GUS expression for both genes was detected in immature siliques where *AtTPPB* was strongly expressed in developing ovules (Fig. 4g, h). High levels of expression for both genes were detected in the silique abscission zone and in the adjacent tissues of the pedicel. GUS expression for *AtTPS1* and *AtTPPB* was low in mature siliques with the exception of tissues near the abscission zone (Fig. 4i, j). Expression of both genes was faint but was associated with the funiculus attached to developing ovules in older siliques (Fig. 4k, l). Although the expression of both genes was detected in older rosettes and stems, the intensity was much lower than in younger tissue (data not shown).

Regulation of *TPS1*, *TPPB* and *TRE* expression in response to carbohydrates and stress

Changes in the expression of *AtTPS1* and *ATTPPB* in response to various chemical and stress treatments were quantified by QPCR. Relative to the H_2O controls, the addition of 0.1 M exogenous sugars, i.e., Suc, Glc, Tre and Fru, to *Arabidopsis* seedlings grown in liquid culture either repressed the expression of *AtTPS1* and *AtTPPB* or the sugars had little or no effect on transcript abundance (Fig. 5a, b, respectively). In general, trehalose did not repress *AtTPS1* and *AtTPPB* to the same extent as treatments with Glc, Suc and Fru. In contrast to soluble sugars, exogenous mannitol, sorbitol and NaCl induced the expression of both trehalose pathway genes and these compounds generally had a greater effect on the induction of *AtTPPB* in comparison to *AtTPS1*. Cold treatments for 24 h also slightly repressed the expression of *AtTPS1* but had little effect on *AtTPPB*. Efforts to learn more about changes in expression of *AtTPS1* and *AtTPSB* by monitoring the responses of GUS activity to chemical and physical stresses were unsuccessful The same sugars, polyols and stress treatment conditions were used as described above but responses to these treatments were not discernible by changes in GUS activity (data not shown).

Discussion

There is growing evidence that metabolites in the trehalose pathway have signaling functions in higher plants and in various microbionts (Paul et al. 2008). Trehalose 6-P modulates hexokinase activity in yeast, thereby suggesting that related functions for this compound exist in higher plants (Blázquez et al. 1993, Avonce et al. 2004). Rates of photosynthetic carbon assimilation in transgenic tobacco leaves were altered by modulating trehalose 6-P levels (Paul and Pellny 2003) and deleting *AtTPS1* resulted in a recessive embryo lethal phenotype which was attributed to a lack of trehalose 6-P (Eastmond et al. 2002). Moreover, the growth inhibition that occurs in plants exposed to exogenous trehalose was shown to be dependent on the accumulation of trehalose 6-P (Schluepmann et al. 2003). Taken together these and other lines of evidence suggest that trehalose 6-P is an important regulatory metabolite in higher plants. Consequently, attaining a better understanding of factors affecting the expression of enzymes involved in trehalose 6-P synthesis and degradation could

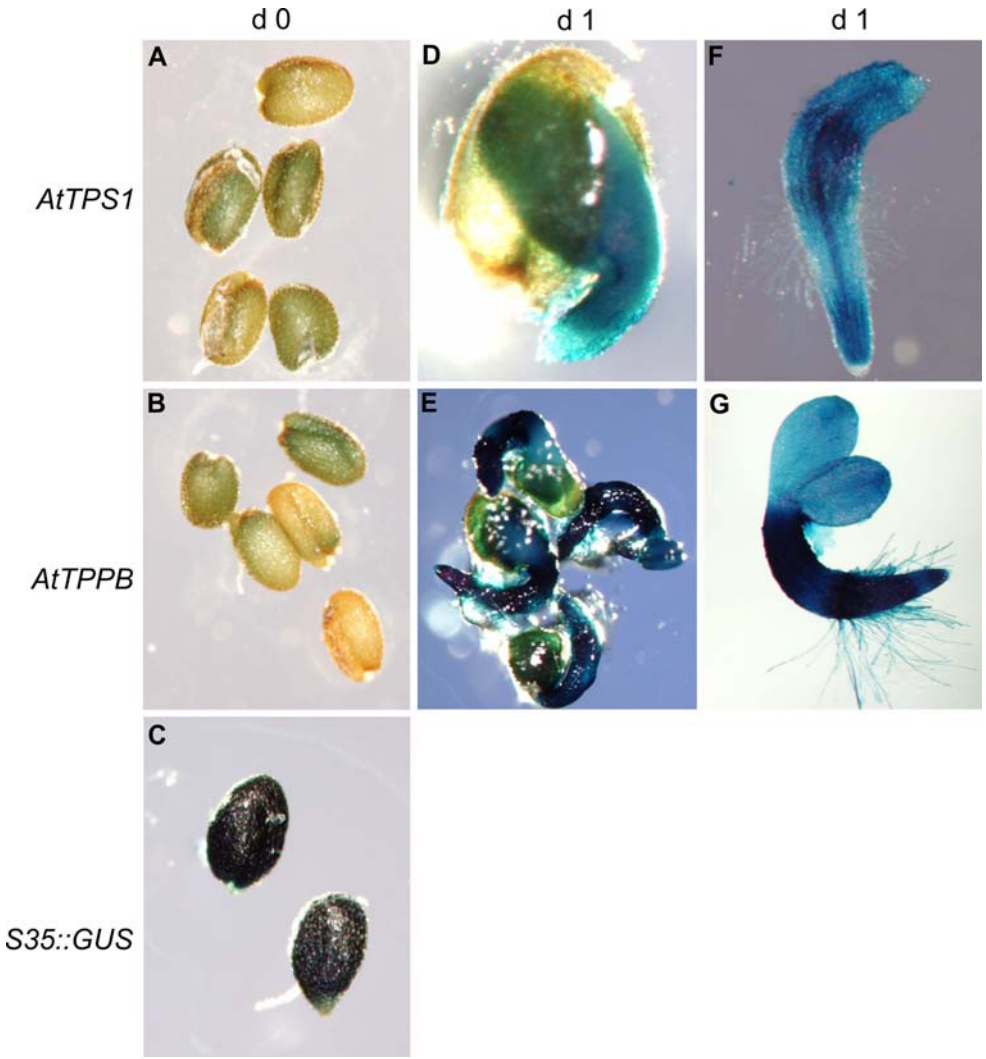

Fig. 1 Expression of *AtTPS1* and *AtTPPB* promoter::*GUS* transgenes in *Arabidopsis* during seed germination. GUS expression was dependent upon promoters for *AtTPS1* (**a**, **d**, **f**), *AtTPPB* (**b**, **e**, **g**) and 35S (**c**). Expression was visible under the seed coat of imbibed seeds on day 0 (**a**, **b**, **c**) and throughout 1-day-old light-grown seedlings (**d**, **e**, **f**, **g**)

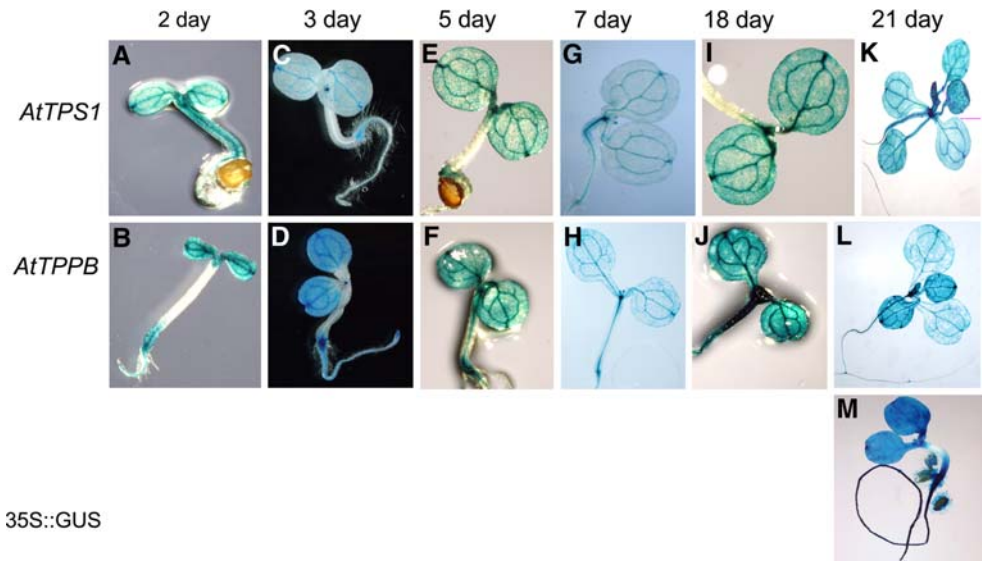

Fig. 2 Expression of *AtTPS1* and *AtTPPB* promoter::*GUS* transgenes in *Arabidopsis* during seedling development. Gus expression was dependent upon promoters for *AtTPS1* (**a**, **c**, **e**, **g**, **i**, **k**), *AtTPPB* (**b**, **d**, **f**, **h**, **j**, **l**) and 35S (M). Images are shown for 2-days (**a**, **b**), 3-days (**c**, **d**), 5-days (**e**, **f**), 7-days (**g**, **h**), 18-days (**i**, **j**) and for 21-days-old (**k**, **l**, **m**) seedlings grown on agar plates in the light

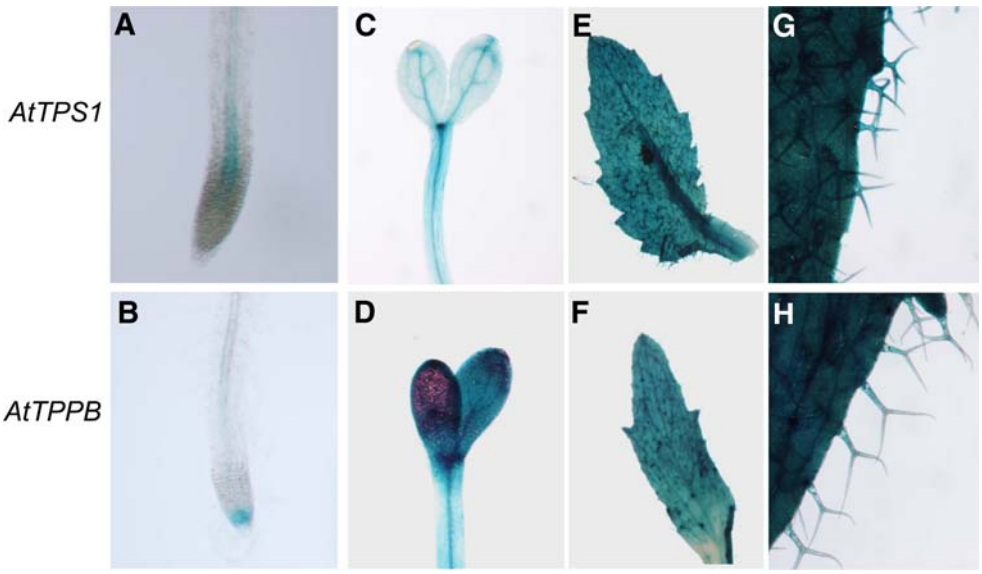

Fig. 3 Expression of the *AtTPS1* and *AtTPPB* promoter::*GUS* transgenes in *Arabidopsis* at various stages of plant development. Gus expression was dependent upon promoters for *AtTPS1* (**a**, **c**, **e**, **g**) and *AtTPPB* (**b**, **d**, **f**, **h**). Expression was detected in roots of 7-days old light grown seedlings (**a**, **b**), in shoots of 7-days-old-etiolated seedlings (**c**, **d**), in rosette leaves of plants grown in soil for 5–6 weeks (**e**, **f**) and in trichomes (**k**, **l** [trichome images were magnified 125x])

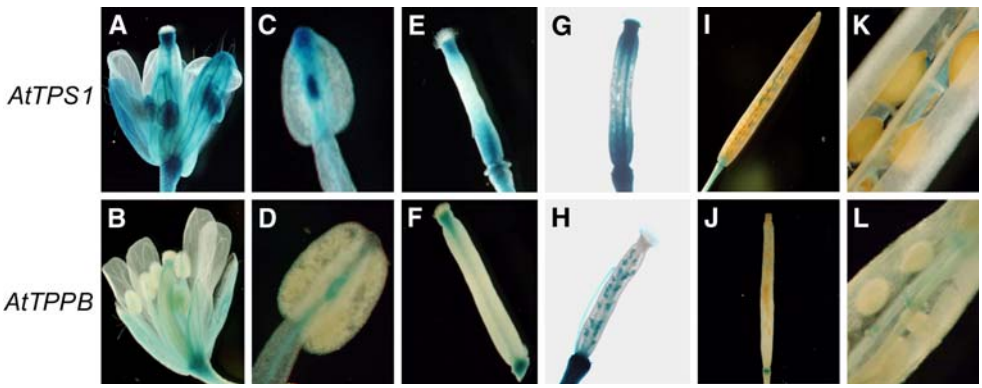

Fig. 4 Expression of the *AtTPS1* and *AtTPPB* promoter::*GUS* transgenes in *Arabidopsis* in floral and reproductive tissues. Gus expression was dependent upon promoters for *AtTPS1* (**a**, **c**, **e**, **g**, **i**, **k**) and *AtTPPB* (**b**, **d**, **f**, **h**, **j**, **l**). Expression was observed in floral organs (**a**, **b**), stamens (**c**, **d**), pistils (**e**, **f**) immature siliques (**g**, **h**) and mature siliques (**i**, **j**, **k**, **l**)

help explain how this compound regulates growth and development in higher plants.

In the current study the expression of *AtTPS1* and *AtTPPB* was concentrated in young, actively dividing tissue with high metabolic activity. Expression of both promoters was readily observed immediately after germination on day 1 but for most plant tissues the expression of both promoters generally became more diffuse later in development as cell division rates abated. The expression of both promoters was clearly evident in the shoot apex and in floral parts, including anthers. This is consistent with the suggestion that these anatomical structures are densely packed with actively dividing cells. Both *AtTPS1* and *AtTPPB* also were expressed in association with vascular tissues of both roots leaves and floral parts. Expression of both promoters was observed in the root immediately below the hypocotyl, in the pedicel just below the silique and in the receptacle of mature flowers. Taken together, the above results suggested that the trehalose pathway was strongly associated with tissues involved in the bulk transfer of organic solutes.

To our knowledge there has been one prior study using Promoter::GUS fusion constructs to examine the expression of genes directly involved in trehalose metabolism. Similar to the present study, van Dijken et al. (2004) studied the expression of *AtTPS1* in *Arabidopsis* using the β-glucuronidase (GUS) reporter gene. We are unaware of prior attempts to study *AtTPPB* by this method. van Dijken et al. (2004) observed *AtTPS1* expression during the torpedo and heart stages of embryogenesis and, in contrast to the present study, GUS activity for *AtTPS1* was absent from all other plant tissues. In the heart-stage of embryo development the expression of *AtTPS1* was concentrated in tissues destined to become the shoot apex. Furthermore, *AtTPS1* in mature ovules was detected at the site of funiculus attachment. van Dijken et al. (2004) do not explain why *AtTPS1* expression could not be visualized by GUS activity other than during embryogenesis but it is likely that prior transformation efforts with *AtTPS1* by van Dijken et al. (2004) were only partially successful.

Recently, Ramon et al. (2009) examined the expression of all seven class II TPS genes from *Arabidopsis* using transformants attached to a promoter-GUS-green fluorescent protein reporter. All of the class II TPS genes were expressed in root tips and in leaf primordia. Expression in other

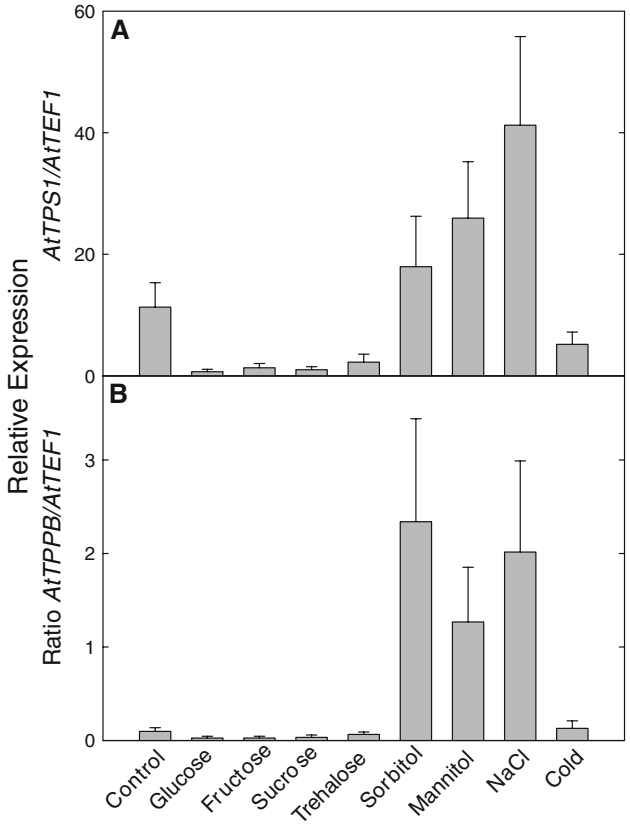

Fig. 5 Effects of various sugars, polyols and stress related treatments on transcript levels of 1-week-old *Arabidopsis* seedlings as determined by QPCR. Seedlings grown in liquid culture were exposed to 100 mM Suc, 100 mM Glc, 100 mM Fru, 100 mM trehalose, 100 mM NaCl, 250 mM sorbitol, 250 mM mannitol or cold (4°C) for 24-h prior to harvest. Transcripts for *AtTPS1* (**a**) and *AtTPPB* (**b**), were measured via real-time QPCR using cDNA synthesized from total RNA. Mean values ± SE are for three independent biological samples

vegetative and reproductive tissues varied for each individual gene. The expression of *AtTPS5* was strongly induced by Suc but genes *AtTPS8*, *AtTPS9*, *AtTPS10* and *AtTPS11* were all repressed by Suc. The expression *AtTPS8* was up-regulated by cytokinin and the expression of several other of the class II TPS genes responded to cytokinin treatment. None of the class II TPS genes had activity in yeast complementation experiments and could not synthesize trehalose 6-P. The authors argued that the regulation of metabolism by the various class II TPS genes was tissue specific or was restricted to individual aspects of plant development.

Changes of transcript levels in response to sugars and abiotic stress treatments

Because trehalose 6-P is thought to be a regulator of carbohydrate metabolism and an important signaling molecule in higher plants, it was of interest to determine how genes in the trehalose pathway responded to exogenous carbohydrates and to stress treatments. In prior experiments (Vogel et al. 1998) observed that *AtTPPB* transcripts were low but detectable in 10 days-old autotrophic and mixotrophic seedlings of *Arabidopsis* grown in agar plates with or without Suc. In a similar study with 8 days-old seedlings, van Dijken et al. (2004) reported that there was a slight induction of *AtTPS1* in response to either Suc or sorbitol. Using hybridization to DNA microarrays, Schluepmann et al. (2004) showed that 5 of 6 *AtTPP* homologs, including *AtTPPB*, were induced up to threefold in response to exogenous trehalose. These authors also reported that *AtTPS1* was induced two to fourfold after a 24 h treatment with trehalose. In the current study transcripts of *AtTPS1* and *AtTPPB* were either repressed or were little affected by exogenous Suc, Glc, Fru or trehalose when transcript abundance was measured by QPCR. However, transcripts for both trehalose biosynthesis genes were induced two to tenfold by sorbitol, mannitol and NaCl. This suggested that the expression of *AtTPS1* and *AtTPPB* may have responded to increased osmotic pressure rather than to the presence of polyols or salt. Because soluble sugars also increase osmotic pressure when exposed to plants, the extent of repression of *AtTPS1* and *AtTPPB* by Suc, Glc, Fru or trehalose should be measured versus sorbitol or mannitol as opposed to the H_2O control. It is not clear why the microarray approach of Schluepmann et al. (2004) for quantifying changes in the expression of *AtTPPB* and *AtTPS1* generated larger responses to Suc than the present study. Unlike the QPCR results, treatment with carbohydrates, salt or cold stress did not alter GUS expression for either *AtTPS1* or *AtTPPB* in this study. However, this would be explained if protein levels of GUS were relatively stable in *Arabidopsis* tissues in comparison to changes in transcript abundance.

Summary

In contrast to earlier reports we show that *AtTPS1* and *AtTPPB* were expressed nearly constitutively in *Arabidopsis* and that the expression of both genes was observed throughout almost all development stages of the plant. However, GUS expression for both genes was pronounced during early seed germination and growth and then diminished in older vegetative tissues. Although GUS expression for *AtTPS1* and *AtTPPB* was diffuse in older leaves and stems, there was a marked concentration of GUS activity for both genes in association with vascular bundles, apical tissue and in the inflorescence. Taken together the above findings indicated that the expression of *AtTPS1* and *AtTPPB* was greatest in young, actively dividing sink tissues, particularly the shoot apex, and was closely associated with the vasculature or other tissues actively involved in solute transport. Both *AtTPS1* and

AtTPPB were repressed slightly by exogenous sugars and were induced by polyols and NaCl. The fact that *AtTPS1* and *AtTPPB* were co-localized in most plant tissues supported the conclusion that these two enzymes function in tandem in the trehalose biosynthesis pathway.

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